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MUHVICH KH, CRISWELL DW, ANDERSON LH, WILSON B, HOWARD RT, MEHM WJ

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Armed Forces Institute of Technology
Division of Altitude and Hyperbaric Physiology CPW-P
Washington, D.C. 20306-6000

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13. ABSTRACT (Maximum 200 words)

This study examined the role of oxygen in amphotericin B-induced killing of Leishmania braziliensis panamensis promastigotes and Candida albicans. In the first phase of the study, we explored the effects of high oxygen tensions on the lethal effects of three reduction-oxidation cycling drugs: amphotericin B, menadione, and phenazine methosulfate. Promastigotes were exposed to the above drugs under normoxic, hyperoxic (100% 02 at 101.3 kPa), or hyperbaric hyperoxic (100% 02 at 253.3 kPa) conditions. After 24 h incubation at 27°C, viable promastigotes stained with fluorescein diacetate and were counted using epifluorescence microscopy. Hyperbaric hyperoxia alone (PO2 = 229 kPa) was as effective as AmB alone (0.2 uM); both killed 80% of the original inoculum. AmB killed more promastigotes in a hyperbaric hyperoxic environment than in normaxic (PO2 = 21.1 kPa) or hyperoxic conditions (PO2 = 91.7 kPa). High oxygen tensions did not alter the lethal effects of either menadione or phenazine methosulfate. In the second phase of the study, the effects of hypoxia on AmB killing in Leishmania and yeast cells were investigated. Leishmania promastigotes were exposed to AmB (0.1 and 1.0 uM) in media with dissolved PO2s of

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22 mmHg (hypoxia) and 150 mmHg (normoxia) for two h at 27°C. Following incubation, promastigotes were stained as above and viable organisms counted. Yeast cells (Candida albicans) were also exposed to AmB (0.25, 0.5, 1.0, 2.0 uM) under the same atmospheric conditions at 35°C. Colony-forming units of yeast cells were counted to assess viability. No significant differences in promastigote viability were seen between air and hypoxic groups. A trend toward decreased killing of yeast cells by AmB under hypoxic conditions was seen. However, the only significant difference was found at 0.5 uM AmB. The results of these studies indicate that lethal effects of hyperbaric hyperoxia are additive to those induced by AmB. In addition, Leishmania promastigotes and intact yeast cells are susceptible to AmB-induced killing in hypoxic environments.

ABSTRACT

13. This study examined the role of oxygen in amphotericin B-induced killing of Leishmania braziliensis panamensis promastigotes and Candida albicans. In the first phase of the study, we explored the effects of high oxygen tensions on the lethal effects of three reduction-oxidation cycling drugs: amphotericin B, menadione, and phenazine methosulfate. Promastigotes were exposed to the above drugs under normoxic, hyperoxic (100% O_2 at 101.3 kPa), or hyperbaric hyperoxic (100% O_2 at 253.3 kPa) conditions. After 24 h incubation at 27°C, viable promastigotes stained with fluorescein diacetate and were counted using epifluorescence microscopy. Hyperbaric hyperoxia alone (PO_2 = 229 kPa) was as effective as AmB alone (0.2 uM); both killed 80% of the original inoculum. AmB killed more promastigotes in a hyperbaric hyperoxic environment than in normoxic (PO_2 = 21.1 kPa) or hyperoxic conditions (PO_2 = 91.7 kPa). High oxygen tensions did not alter the lethal effects of either menadione or phenazine methosulfate. In the second phase of the study, the effects of hypoxia on AmB killing in Leishmania and yeast cells were investigated. Leishmania promastigotes were exposed to AmB (0.1 and 1.0 uM) in media with dissolved PO_2s of 22 mmHg (hypoxia) and 150 mmHg (normoxia) for two h at 27°C. Following incubation, promastigotes were stained as above and viable organisms counted. Yeast cells (Candida albicans) were also exposed to AmB (0.25, 0.5, 1.0, 2.0 uM) under the same atmospheric conditions at 35°C. Colony-forming units of yeast cells were counted to assess viability. No significant differences in promastigote viability were seen between air and hypoxic groups. A trend toward decreased killing of yeast cells by AmB under hypoxic conditions was seen. However, the only significant difference was found at 0.5 uM AmB. The results of these studies indicate that lethal effects of hyperbaric hyperoxia are additive to those induced by AmB. In addition, Leishmania promastigotes and intact yeast cells are susceptible to AmB-induced killing in hypoxic environments.

INTRODUCTION

1. Objectives

This study characterized the effects of oxygen tensions on the lethal effects of amphotericin B and other reduction-oxidation cycling drugs. Leishmania braziliensis panamensis promastigotes and Candida albicans yeasts cells were used to test the efficacy of hyperoxia and hyperbaric hyperoxia in augmenting the killing of antimicrobial agents. The specific objectives of this study were as follows:

a) to determine the minimal oxygen tension that was toxic for Leishmania promastigotes.

b) to characterize the combined lethal effects of high oxygen tensions (hyperoxia and hyperbaric hyperoxia) and antimicrobials against Leishmania promastigotes

c) to use low oxygen tensions (hypoxia) to determine if lethal effects of AmB against Leishmania and yeast cells were oxygen dependent

It was the intention of the original protocol to carry the findings of the in vitro work into macrophage cells lines and eventually a mouse model of leishmaniasis. However, oxygen tensions that combined with AmB to increase lethal effects in promastigotes exceeded the minimal toxic limits for macrophage cells and mice.

2. Findings

A cumulative, substantive and comprehensive statement and discussion of research background, rationale, material, methods and scientific significance can be found in the appendices. The major findings of this study are as follows:

1) High oxygen tensions, both alone and by themselves exerted toxic effects on Leishmania promastigotes. Hyperoxia inhibited growth and hyperbaric hyperoxia killed a substantial amount of promastigotes.

2) The lethal effects of amphotericin B could be augmented by hyperbaric hyperoxia.

3) The lethal effects of menadione and phenazine methosulfate were not altered by high oxygen tensions.

4) Hypoxia does not decrease the efficacy of AmB against either Leishmania promastigotes or C. albicans cells.

3. Presentations and Publications

A portion of this study was presented at the 31st Interscience Conference on Antimicrobials Agents and Chemotherapy held September 29 - October 2 1991 in Chicago, Illinois. The paper presented was published as abstract no 682 in the Program and Abstracts for the ICAAC meeting.

Another portion of this study was presented at the 25th Annual

meeting of the Undersea and Hyperbaric Medical Society. The paper was published as abstract no. 27 in the Program and Abstracts of the 1992 Undersea and Hyperbaric Medical Society Annual Scientific Meeting. The UHMS meeting was held 23-27 June 1992.

4. Appendices

Appendix A - Abstract no 682 which was presented at the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy held form September 29 to October 2, 1991

Appendix B - Draft of a research paper which was submitted to the journal Undersea Biomedical Research

Appendix C - Abstract no 27 which was presented at the 25th Annual Undersea and Hyperbaric Medical Society Scientific meeting on June 24th.

Appendix D - Draft of a research paper which was submitted to The Trauma Journal

APPENDIX A

Official Abstract Form

Instructions: Complete this form and submit it for receipt by 26 April 1991. *Only this original form is acceptable (no photocopies).* Additional forms are available from the ASM Meetings Department. Refer to the sample abstract (page xviii) for style. Type the title (initial capitals only) first; then list all authors (all capital letters), with an asterisk for the person delivering the paper; and then list institutions and short addresses (do not give departments, divisions, buildings, etc.). See "Preparation of Abstracts" for additional required enclosures. *Note: Any poorly prepared abstract unsuitable for direct reproduction will be reviewed by the ICAAC Committee; however, if accepted for presentation at the conference, it will not be published in the* Program and Abstracts.

Oxidative Stress Enhances Leishmanicidal Effects of Amphotericin B.

K. H. MUHVICH*, D. W. CRISWELL, and W. J. MEHM. Armed Forces Institute of Pathology, Washington, DC.

Oxidative damage causes amphotericin B (AmB)-induced killing of yeast cells. Hyperoxia promotes the formation of toxic oxygen species and may act in concert with AmB to increase killing of other susceptible microorganisms, such as Leishmania. To test this hypothesis, L. braziliensis panamensis (WRAIR 676) promastigotes were exposed to hyperoxia alone [100% O_2 at total pressures of 101.3 or 253.3 kilopascals (kPa)], or in combination with 0.2 uM AmB. Parasites exposed to 5% or 21% O_2 at 101.3 kPa served as controls. After incubation for 24 h at 27°C, viable parasites were stained with fluorescein diacetate and counted using epifluorescence microscopy. Hyperoxia at 253.3 kPa was Leishmanicidal; approximately 80% of the original inoculum (10^6) was killed. Hyperoxia (253.3 kPa) plus AmB significantly reduced ($p < 0.05$) the number of viable parasites as compared to hyperoxia (101.3 kPa) and AmB. Hyperoxia or prooxidant compounds may combine with low doses of AmB to effectively kill Leishmania promastigotes.

Category D

Subject category: From the list of subject categories on p. xxv, choose the most appropriate description of the paper's content and enter the letter in the box above. *Example:* "Comparison of Methods for the Serodiagnosis of Herpes Simplex Virus Infections" should be classified as Category G-1.

Complete the following:

1. ☒ This material will not have been published or presented at a scientific meeting before 29 September 1991. (This includes international meetings and congresses.)

2. Full name and professional mailing address of the author who will present the paper. Kenneth H. Muhvich, Ph.D., Armed Forces Institute of Pathology, Division of Altitude and Hyperbaric Physiology, CPW-P, Washington, DC 20306-6000

Telephone no. 202-576-2868 Fax no. 202-576-2164

3. Complete checklist on p. xxviii before submitting abstract. Abstracts submitted via fax machine will not be accepted by the Program Committee.

4. Mail abstract to ICAAC Abstracts, American Society for Microbiology, 1325 Massachusetts Ave., N.W., Washington, DC 20005-4171.

APPENDIX B

Hyperbaric Hyperoxia Enhances the Lethal Effects of Amphotericin B in *Leishmania braziliensis panamensis*.

Kenneth H. Muhvich, Darrell W. Criswell, and William J. Mehm*

Division of Altitude and Hyperbaric Physiology, Armed Forces Institute of Pathology, Washington, DC 20306-6000.

Running Head: HBO ENHANCES LETHAL EFFECTS OF AMB

A portion of this study was published as paper no. 682 in the Program and Abstracts of the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy. The ICAAC meeting was held September 29 - October 2 1991 in Chicago, IL.

*Author to whom correspondence should be addressed.

Military Status: Both Darrell W. Criswell and William J. Mehm are Majors in the Biomedical Sciences Corps of the United States Air Force.

ABSTRACT

Muhvich KH, Criswell DW, Mehm WJ. Hyperbaric hyperoxia enhances the lethal effects of amphotericin B (AmB) in Leishmania braziliensis panamensis. Undersea Biomed Res 199 ; ..( ): - .---- Leishmania braziliensis panamensis promastigotes were exposed in vitro to amphotericin B, menadione, or phenazine methosulfate under normoxic conditions. Promastigotes were also exposed to hyperoxia alone [100% O_2 at total pressures of 101.3 or 253.3 kilopascals (kPa)], or combined with drugs. After incubation for 24 h at 27°C, viable promastigotes stained with fluorescein diacetate and were counted using epifluorescence microscopy. Hyperbaric hyperoxia alone (PO_2 = 229.3 kPa) was as effective as AmB alone (0.2 uM); both reduced the number of viable promastigotes by approximately 80%. In addition, AmB in a hyperbaric hyperoxic environment killed more promastigotes than AmB in normoxic (PO_2 = 21.1 kPa) or hyperoxic conditions (PO_2 = 91.7 kPa). Finally, AmB in hyperbaric hyperoxia killed significantly more promastigotes than hyperbaric hyperoxia alone. High oxygen tensions did not alter the lethal effects of either menadione or phenazine methosulfate. In conclusion, the lethal effects of low dose AmB in Leishmania promastigotes were augmented by hyperbaric hyperoxia in vitro.

Key words: hyperbaric hyperoxia
oxidative stress
Leishmania
promastigotes
amphotericin B
phenazine methosulfate
menadione
redox cycling drugs

INTRODUCTION

Leishmania are protozoan parasites which cause debilitating disease in tropical regions of South America, Asia, Africa and the Middle East. Leishmaniases are more abundant than was previously thought. Twelve million cases exist world-wide and 350 million people are estimated to be at risk for acquiring these infections (1). In endemic areas, leishmaniases are transmitted to human hosts through bites of phlebotamine sand flies infected with the extracellular promastigote form of the parasite. Recently, attention has been focused on this disease in the United States because seven cases of visceral leishmaniasis were identified in military personnel returning from countries surrounding the Persian Gulf (2).

Pentavalent antimony is the chief chemotherapeutic agent used to treat the clinical forms of leishmaniases: visceral, cutaneous, and mucocutaneous (3). Amphotericin B (AmB) is used as a second-line drug for treatment of nonresponsive leishmaniases (3,4). AmB is a polyene antibiotic primarily used to treat fungal infections and its fungicidal effects are mediated by an oxygen-dependent mechanism (5,6,7). Because AmB auto-oxidizes in the presence of molecular oxygen (8,9), reactive oxygen intermediates formed are thought to be responsible for the fungicidal effects of AmB. Pro-oxidants, such as ascorbic acid (10) and hyperbaric hyperoxia (11) enhance the lethal effects of AmB against Candida albicans.

A proposed mechanism for the lethal effects of AmB is formation of superoxide anion when the drug undergoes auto-oxidation (9). Superoxide anion is also produced when oxygen accepts an electron from drug free radicals. Electron carriers, such as menadione (MDN) and phenazine methosulfate (PMS), generate superoxide anions during redox cycling reactions and induce lethal effects in Leishmania promastigotes (12). However, the combined effects of pro-oxidants such as hyperoxia and redox cycling agents against Leishmania have not been characterized.

Leishmania promastigotes may be more vulnerable than fungi to oxidative stress induced by hyperoxia combined with AmB, MDN, or PMS. The major antioxidant defense of Leishmania spp. promastigotes is superoxide dismutase. Superoxide anions formed during AmB auto-oxidation (8), during MDN or PMS redox-cycling reactions, or under hyperoxic conditions (13) undergo rapid dismutation catalyzed by superoxide dismutase to form hydrogen peroxide (H_2O_2). In most eukaryotic cells the H_2O_2 is then detoxified by catalase or glutathione peroxidase. However, low levels of these enzymes are present in Leishmania spp. promastigotes (14,15). Thus, H_2O_2 may accumulate in a sufficient concentration in promastigotes under oxidative stress to cause toxic effects, such as enzyme inactivation. In fact, studies have shown that H_2O_2 is directly toxic to Leishmania spp. in vitro (15,16,17) and inactivates L. tropica superoxide dismutase (14). Therefore, it is likely that Leishmania promastigotes are vulnerable to even the low

concentrations of H_2O_2 (nM) generated under hyperoxic conditions (18). The purpose of this study was twofold: to determine the tolerance of L. braziliensis panamensis promastigotes for high doses of oxygen and to determine if drug-induced killing of promastigotes could be augmented by concomitant exposure to high oxygen tensions.

MATERIALS AND METHODS

Test organism

L. braziliensis panamensis (WRAIR 676) promastigotes were obtained from Dr. Ronald Anthony, Department of Pathology, University of Maryland, Baltimore, MD. L. braziliensis panamensis promastigotes were maintained at 27°C in Dulbeco's modified Eagle medium (DMEM), Formula No. 78-0176P, containing 10% fetal bovine serum (GIBCO BRL, NY). Prior to each experiment, actively growing cell suspensions were diluted 1:10 and incubated as above for 72 h. Promastigotes were then pelleted by centrifugation at 250 x g for 10 min and the supernatant was discarded. The pellet was washed twice with phosphate buffered saline (PBS), adjusted to 10^7 organisms/ml, and added to oxygenated media with or without AmB such that the final concentration of promastigotes was 10^6/ml.

Drugs and oxygen exposures

Amphotericin B, menadione bisulfite, and phenazine methosulfate were purchased from Sigma Chemical Co., St. Louis, MO. The formulation of AmB contained sodium deoxycholate (35%) to facilitate dispersion of AmB in water. AmB, MDN, and PMS were dissolved in distilled water to yield 1.0 M stock solutions. Stock solutions of drugs were filter sterilized and stored under nitrogen at -20°C to prevent auto-oxidation. Sodium deoxycholate used alone, in the concentration used to solubilize AmB, did not affect promastigote viability. The stock solutions of drugs were diluted in DMEM to yield the following concentrations for experiments:

AmB (0.2 uM), MDN (9.4 uM), and PMS (6.3 uM). Preliminary results showed that those were the lowest concentrations of each drug which induced minimal lethal effects in L. braziliensis panamensis promastigotes.

Dissolved oxygen tensions of media with or without drugs were adjusted using vacuum-assisted diffusion (19). Each desired oxygen atmosphere (Table 1) was achieved in a controlled atmosphere glove box (Labconco Corp., Kansas City, MO.) using an oxygen regulator, and confirmed using a model 1100 medical gas analyzer (Perkin-Elmer Corp., Pomona, CA). Media were pipetted into a 0.5 L bottle and dissolved gases were removed using a vacuum pump connected to the glove box. Each desired dissolved oxygen tension was achieved by swirling bottled media while under negative pressure, followed by venting the bottle into the glove box atmosphere. The vacuum/venting procedure was repeated several times over a 5 min period. Achievement of the target oxygen tension was confirmed by injecting oxygenated media into a model 170 pH/blood gas analyzer (Corning Medical, Medfield, MA).

Promastigote viability

After the target oxygen tension was achieved in the culture media, one part promastigote suspension was added to nine parts oxygenated media with or without drug [AmB, MDN, or PMS] (Fig. 1.). The final concentration of promastigotes was 1 X 10^6/ml. Five ml aliquots were pipetted into 25 cm^2 tissue culture flasks. Depth of

the culture media in the flasks was approximately 3 mm to facilitate oxygen diffusion. Flasks were incubated in air (normoxia, 21%O_2, 101.3 kilopascals), normobaric hyperoxia (100% O_2, 101.3 kPa), or hyperbaric hyperoxia (100% O_2, 253.3 kPa) at 27°C±1°C for up to 24 hr (Table 1). Normobaric hyperoxic cultures were maintained in controlled atmosphere culture chambers (Bellco Inc., Vineland, NJ). Hyperbaric hyperoxia was achieved and maintained in a model 615 HP hyperbaric chamber (Bethlehem Corp., Bethlehem, PA). After incubation, promastigote suspensions were removed from flasks, pelleted and washed with PBS as above. Promastigotes were then stained with 5 ug/ml fluorescein diacetate (FDA) for five min. Viable organisms stained green and were counted on a hemacytometer using an epifluorescent microscope. Suspensions of promastigotes which failed to stain with FDA were placed in fresh media and maintained in a normoxic environment (PO_2 = 21 kPa) for 72 hours. In these cultures, no viable organisms were seen, i.e. cellular swelling was present in all organisms and no flagellar motility was observed.

Statistical Analysis

Data were analyzed using a one-way ANOVA (analysis of variance). The Kruskal-Wallis test was used to determine if significant differences existed between experimental groups. Data were considered significantly different at $P < 0.05$.

RESULTS

Effects of oxygen on AmB-induced killing of promastigotes

As oxygen tensions in culture media increased, numbers of viable L. braziliensis panamensis promastigotes decreased (Fig. 2). Growth stasis was seen in hyperoxia-exposed promastigote cultures, while hyperbaric hyperoxia induced lethal effects. Normoxic control cultures (PO_2 = 21.1 kPa) grew 0.5 $\log_{10}$ over 24 h. Numbers of viable promastigotes in normobaric hyperoxic control cultures (PO_2 = 91.7 kPa) were not significantly different from the original inoculum. Yet, the original inoculum of viable promastigotes was decreased by 80% in hyperbaric hyperoxia ($P < 0.05$). The PO_2 in these cultures had been increased to 229.3 kPa via a hyperbaric chamber. Promastigote viability was decreased to the same extent in hyperbaric hyperoxia control cultures as in normoxic AmB-treated cultures.

Achievement of the maximal oxygen tension induced by hyperbaric oxygenation was necessary before interactive effects with AmB were observed. There was no decrease in viability when promastigotes were exposed to AmB under hyperoxic conditions as compared to normoxic AmB-treated cultures. However, AmB-induced killing of promastigotes was significantly increased by concomitant exposure to hyperbaric hyperoxia as compared to normoxic and hyperoxic conditions. Significant lethal effects for AmB under hyperbaric hyperoxic conditions were not found when promastigote cultures were exposed for less than two hours.

Effects of oxygen on menadione and phenazine methosulfate-induced killing of promastigotes

As seen in Fig. 2, both menadione and phenazaine methosulfate induced lethal effects in promastigotes which were not augmented by high oxygen tensions. Each redoxy cycling drug reduced the original inoculum by approximately 0.5 $\log_{10}$ in normoxic conditions over 24 h. Exposure of promstigotes to MDN and PMS under hyperoxic or hyperbaric hyperoxic conditions did not further decrease the numbers of viable organisms as compared to normoxic conditions.

DISCUSSION

The results of this study showed that prolonged exposure to high oxygen tensions is toxic for L. braziliensis panamensis promastigotes. Normobaric hyperoxia inhibited growth of promastigotes, while hyperbaric hyperoxia actually killed them. It is probable that promastigotes suffered sublethal and lethal effects due to the increased flux of oxygen-derived free radicals under hyperoxic and hyperbaric hyperoxic conditions.

The study also examined the combined effects of high oxygen tensions and drugs capable of generating oxygen free radicals. AmB-induced killing of promastigotes in normobaric hyperoxia was not different from AmB-induced killing in normoxic conditions. This comparison serves to underscore the conclusion that normobaric hyperoxia inhibited growth of promastigotes, but was not synergistic with AmB-induced lethal effects. The combined effects of hyperbaric hyperoxia and AmB were additive in that they increased promastigote killing over that seen in normobaric hyperoxia and AmB. In contrast, neither normobaric hyperoxia nor hyperbaric hyperoxia increased promastigote killing by MDN or PMS. The flux of oxygen-derived free radicals from AmB, MDN, and PMS may have been maximal under normoxic conditions, so that no increase in promastigote killing was seen under normobaric hyperoxic conditions. Under hyperbaric hyperoxic conditions, the reducing equivalents necessary for continuous redox cycling of MDN and PMS may not have been available. The lethal effects observed with

hyperbaric hyperoxia alone may have been due to lipid peroxidation. AmB auto-oxidation does not result in lipid peroxidation (8), but does result in the formation of carbon centered free radicals (20). These carbon-centered radicals react with oxygen to eventually form superoxide anion (20). Dismutation of superoxide to H_2O_2 and formation of other reactive oxygen metabolites could result in lethal effects to Leishmania. We conclude that hyperbaric hyperoxia, AmB, and the redox cycling drugs, MDN and PMS may kill Leishmania by different mechanisms.

It is known that oxygen is required for the lethal effects of AmB against C. albicans (6) and Trypanosoma cruzi (8) a protozoan parasite related to Leishmania. However, the mechanism(s) for the lethal effects of AmB have not been determined. Sokol-Anderson et al. (6) showed that by lowering the O_2 tension, the efficacy of AmB is reduced. In their work, AmB-induced lysis of C. albicans protoplasts was reduced by 80% in relatively hypoxic conditions (PO_2 = 40 mmHg) as compared to AmB in air. A PO_2 of 40 mmHg approximates normal host tissue oxygen tensions. However, in our study, AmB-induced killing of Leishmania promastigotes was not altered under the same conditions as compared to air.

The results of our study are consistent with the work of Gudewicz et al. in C. albicans (11). They found that neither the minimal inhibitory concentration (MIC) nor the minimal candidacidal concentration (MCC) of AmB for C. albicans were altered by exposure

to a PO_2 of 900 mmHg for 24 h. The MIC is lowest concentration of an antifungal agent which will cause growth to cease in culture and the MCC is the lowest concentration of an antifungal agent which will kill fungal cells. The lethal effects of short term (90 min) hyperbaric hyperoxia (PO_2 = 1800 mmHg) were additive to the lethal effects of AmB. Both the MIC and MCC of AmB for C. albicans were decreased from 0.39 ug/ml in air to 0.10 ug/ml in hyperbaric hyperoxia. In our study, an additive lethal effect was also seen when Leishmania promastigotes were exposed to AmB in hyperbaric hyperoxia (PO_2 = 1720 mmHg). However, short term lethal effects of hyperbaric hyperoxia were not observed. The results of these two studies indicate that PO_2s in excess of 1700 mmHg may be required to enhance lethal effects of AmB in yeast cells and protozoan parasites. Such high oxygen tensions can be attained in wound tissue using hyperbaric oxygenation. Oxygen tensions ranging from 1,000 to 1,700 mmHg have been reported in wounds of human patients (21) during hyperbaric oxygen treatment (100% O_2 at 2.4 ATA, PO_2 = 243 kPa). In this study, however, the duration of exposure of parasites to hyperbaric hyperoxia was much longer (24 h) than could be tolerated by a human patient. Interestingly, no significant detrimental effects were seen in promastigote cultures exposed to AmB under hyperbaric hyperoxic conditions for a single two-hour period.

Thus, it is clear that high partial pressures of oxygen (e.g. >240 kPa) might possibly exert detrimental effects in fungi and

protozoan parasites treated with AmB in vivo. The effects of repeated intermittent HBO treatments on fungi and parasites treated with AmB in suitable animal models are yet to be determined.

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TABLE 1. Experimental atmospheres

Condition	$\%O_2$	Pressure[a]	Dissolved PO_2[b]	
			kPa	mmHg
normoxia	21%	101.3	21.1	158
normobaric hyperoxia	100%	101.3	91.7	688
hyperbaric hyperoxia	100%	253.3	229.3	1720

[a]Total pressure of gases expressed as kilopascals (kPa).

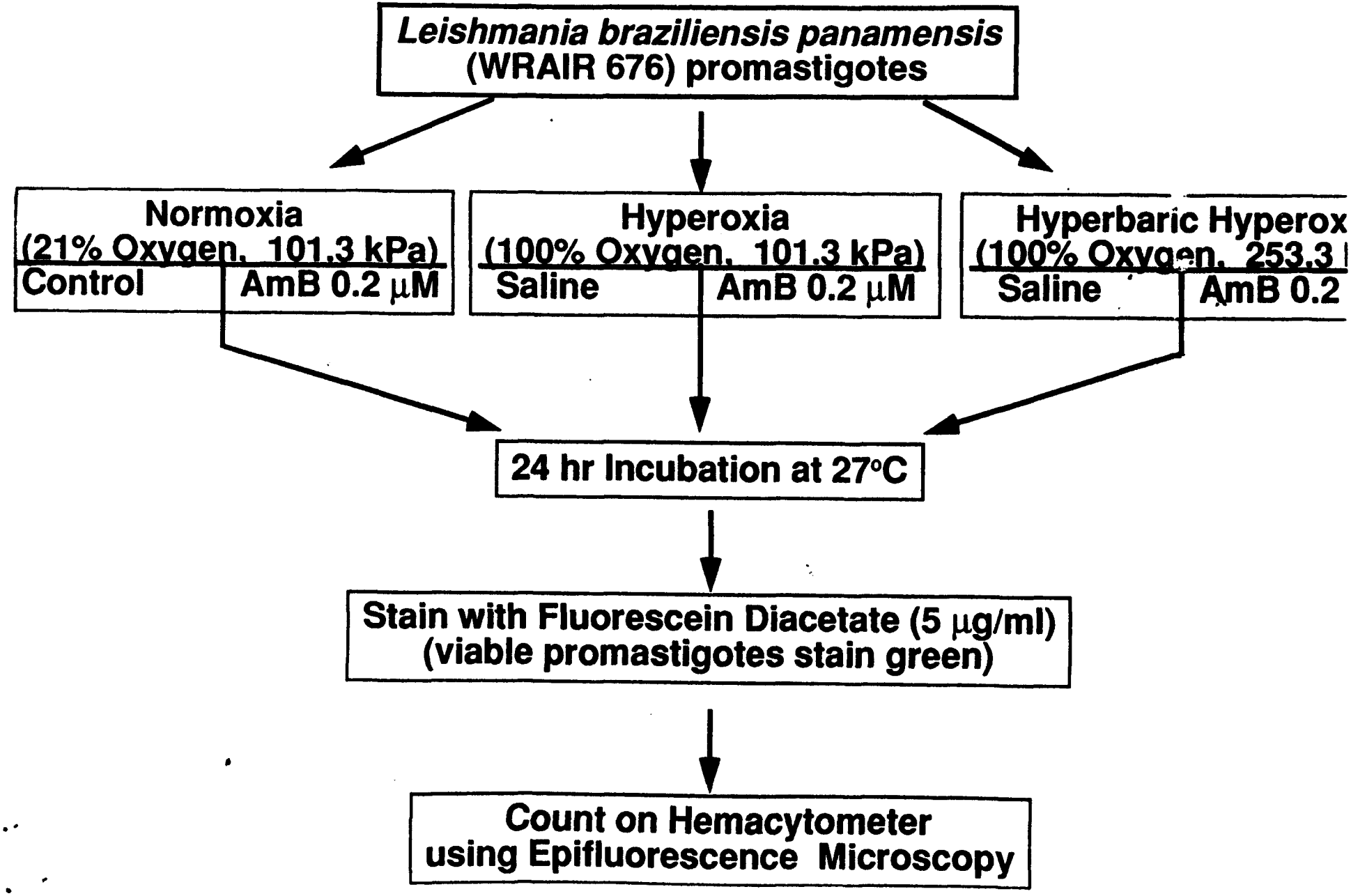
[b]Partial pressure of oxygen as measured in culture media.

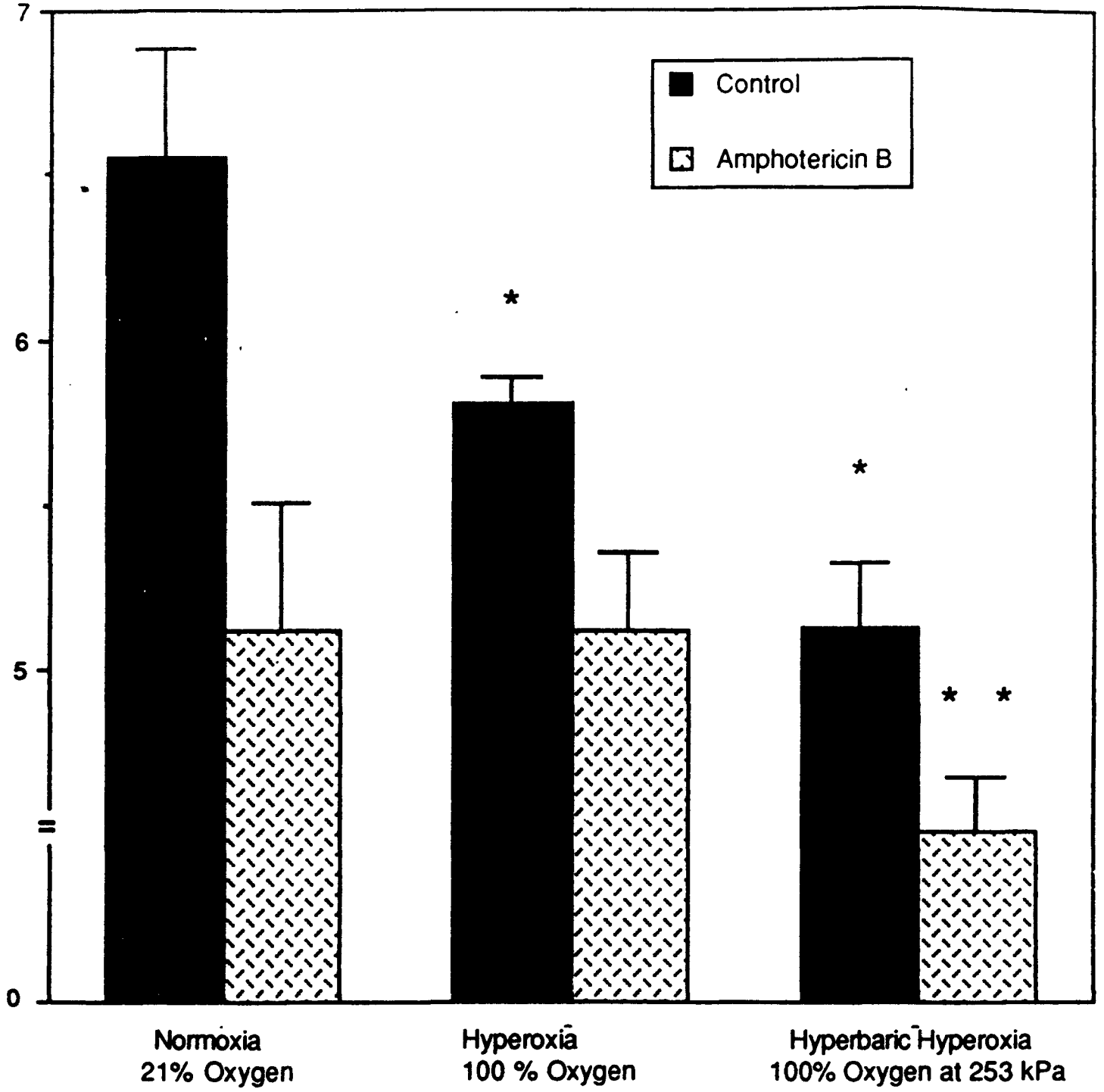
FIGURE LEGENDS

FIG. 1. Flow diagram for exposure of Leishmania braziliensis panamensis promastigotes to drugs and various oxygen tensions.

FIG. 2. Viability of Leishmania braziliensis panamensis promastigotes after 24 h exposure to AmB (0.2 uM), MDN (9.4 uM), and PMS (6.3 uM) in different oxygen environments. The initial concentration of promastigotes was 1 x 10^6/ml, which is denoted as a dashed line (---). Data are from three independent experiments and vertical bars indicate mean number of viable promastigotes±SD. Asterisk (*) denotes significant difference from normoxic control ($p < 0.05$). Double asterisk (**) denotes significant difference from AmB in normoxic and hyperoxic conditions and from hyperbaric hyperoxic control ($p < 0.05$).

Experimental Design





Oxygen Exposure

/iability of *Leishmania braziliensis panamensis* promastigotes after 24 h

APPENDIX C

HYPOXIA AND LETHAL EFFECTS OF AMPHOTERICIN B (AmB) IN LEISHMANIA BRAZILIENSIS PANAMENSIS PROMASTIGOTES. K.H. Muhvich, D.W. Criswell, and W.J. Mehm. Division of Altitude and Hyperbaric Physiology, The Armed Forces Institute of Pathology, Washington, D.C. 20306-6000.

The mechanism responsible for amphotericin B-induced lethal effects in fungi is thought to be oxidation-dependent. AmB-induced lysis of Candida albicans protoplasts is reduced in hypoxic conditions. Our recent work indicates that AmB-induced killing of Leishmania, a protozoan parasite, is enhanced by hyperbaric hyperoxia. The purpose of this study was to evaluate effects of AmB on L. braziliensis panamensis promastigote viability under acute (2 hr) and chronic (18 hr) hypoxic conditions. To this end, promastigotes were exposed to AmB in culture medium with extremely low oxygen tensions. In the acute experiment, promastigotes were exposed to AmB (0.1 and 1.0 uM) in media with dissolved PO_2s of 4.7 kPa (hypoxic) and 19.9 kPa (normoxic). Promastigotes were stained with fluorescein diacetate and viability was assessed using fluorescence microscopy. No differences in viability were seen between hypoxic and normoxic groups at AmB concentrations of 0.1 uM (42% alive) or 1.0 uM (0.2% alive). In the chronic experiment, promastigote cultures containing 0.1 uM AmB were subjected to a prolonged hypoxic exposure (2.4 kPa oxygen). No difference in numbers of viable organisms was seen between normoxic and hypoxic groups. Thus, low oxygen tensions did not diminish AmB-induced killing of Leishmania promastigotes. Our preliminary data indicate that the mechanism responsible for AmB-induced lethal effects is not oxygen-dependent.

APPENDIX D

Hypoxia and Amphotericin B (AmB)-induced Killing of *Leishmania braziliensis panamensis* and *Candida albicans*.

Kenneth H. Muhvich, Loraine H. Anderson, Bernard Wilson, Robert T. Howard, and William J. Mehm*

Running Head: HYPOXIA AND LETHAL EFFECTS OF AMB

A portion of this study was published as paper no. 27 in the *Program and Abstracts of the 1992 Undersea and Hyperbaric Medical Society Annual Scientific Meeting*. The UHMS meeting was held 23-27 June 1992.

*Author to whom correspondence should be addressed.

Military Status: Both Loraine H. Anderson and William J. Mehm are Majors in the Biomedical Sciences Corps of the United States Air Force. Bernard Wilson is a Staff Sergeant in the United States Air Force. Robert T. Howard is a Midshipman in the United States Navy.

Abstract

Muhvich KH, Wilson B, Howard RT, Anderson LH, Mehm WJ. Effect of hypoxia on amphotericin B (AmB)-induced killing of Candida albicans and Leishmania braziliensis panamensis. J Hyperbaric Med 199 ; ( ): . ----- The mechanism responsible for amphotericin B-induced lethal effects in fungi is thought to be oxygen-dependent. The purpose of this study was to compare the effects of hypoxia on AmB-induced killing of L. braziliensis panamensis and Candida albicans. Leishmania promastigotes and Candida yeast cells were exposed to AmB in culture media with low oxygen tensions and viability assessed following incubation. Promastigotes were exposed to AmB (0.1 and 1.0 uM) in media with dissolved PO_2s of 22 mmHg (hypoxia) and 150 mmHg (normoxia) for two h at 27°C. Following incubation, promastigotes were stained with fluorescein diacetate and their viability was assessed using fluorescence microscopy. Yeast cells were also exposed to AmB (0.25, 0.5, 1.0, or 2.0 uM) under the same atmospheric conditions at 35°C. Colony-forming units of yeast cells were counted to assess viability. No significant differences in promastigote viability were seen between air and hypoxic groups at AmB concentrations of 0.1 uM (42% vs 22% alive) or 1.0 uM (0.2% for both groups). A trend toward decreased killing of yeast cells by AmB under hypoxic conditions was observed. The only statistically significant difference was found at 0.5 uM AmB. These experiments indicate that Leishmania promastigotes and intact yeast cells are susceptible to AmB-induced killing in hypoxic environments.

Key words: hypoxia amphotericin B Candida albicans Leishmania braziliensis promastigote yeast

Introduction

The polyene antibiotic amphotericin B (AmB) is toxic to fungal cells and protozoan parasites. Oxygen is required for the lethal effects of AmB against Candida albicans (1) and Trypanosoma cruzi (2). The mechanism of action for lethal effects of AmB on cells is thought to be mediated by oxidation-dependent events which produce reactive oxygen intermediates [ROI] (3). ROI are formed when AmB auto-oxidizes in the presence of molecular oxygen (2,4). ROI damage may be responsible for the fungicidal effects of AmB.

Sokol-Anderson et al. (1) have shown that the lysis of Candida albicans protoplasts (yeasts lacking cell walls) is dependent on the ambient partial pressure of oxygen (PO_2). Under normoxic conditions, 2.0 uM AmB induced 100% lysis of protoplasts. However, under hypoxic conditions the same concentration of AmB caused only 20% lysis of protoplasts. When ambient oxygen tensions are in the hypoxic range (i.e. less than 30 mmHg), auto-oxidation of AmB may produce an insufficient flux of ROI to cause lethal effects. Therefore, the optimal lethal effects induced by AmB may require oxygen tensions in excess of those found in infected tissues.

Leishmania are protozoan parasites which are closely related to

Trypanosoma sp. and are readily killed by AmB (5,6). They are also susceptible to the toxic effects of ROI (7,8) and oxidative stress induced by high oxygen tensions (9). We recently found that promastigotes, the culture form of the parasite, failed to grow in a hyperoxic environment (9). In addition, a significant number of promastigotes were killed in hyperbaric hyperoxia. Interestingly, AmB-induced lethal effects were increased in hyperbaric hyperoxia (9). An additive lethal effect was observed when L. braziliensis panamensis promastigotes were exposed to 0.1 uM AmB in media with a dissolved PO_2 of 1720 mmHg for 24 h. Hyperbaric hyperoxia also enhances the lethal effects of AmB against C. albicans (10). Gudewicz et al. found that when C. albicans were exposed to AmB in an environment with a PO_2 of 1800 mmHg for 90 min, the lethal effects of AmB were increased four-fold (10). In summary, both C. albicans and L. braziliensis panamensis are more susceptible to the lethal effects of AmB in hyperbaric hyperoxia than in normoxia.

We hypothesize that optimal lethal effects of AmB against susceptible organisms probably occurs in normoxic environments, because hypoxia has been shown to reduce the lethal effects of AmB in yeast lacking cell walls. The purpose of this study was to compare the effects of hypoxia on AmB-induced killing of Leishmania promastigotes to C. albicans cells in order to determine if Leishmania killing by AmB is oxygen dependent.

Materials and Methods

Test organisms

L. braziliensis panamensis (WRAIR 676) promastigotes were obtained from Dr. Ronald Anthony at the University of Maryland (Baltimore, MD). Promastigotes were maintained at 27°C in Dulbeco's modified Eagle medium (DMEM, Gibco formula no. 78-0176P) containing 10% fetal bovine serum (Gibco BRL, NY). Prior to each experiment, actively growing cell suspensions were diluted 1:10 and incubated as above for 72 h. Promastigotes were then pelleted by centrifugation at 250 X g for 10 min and the supernatant discarded. The pellet was washed twice with phosphate buffered saline, adjusted to 10^7 organisms/ml, and added to oxygenated media with or without AmB, such that the final concentration of promastigotes was 10^6/ml.

Candida albicans ATCC 32354 was obtained from the American Type Culture Collection (Rockville, MD) and maintained on Sabouraud's dextrose agar. C. albicans cells were grown for 16 h, suspended in Sabouraud' dextrose broth medium, and adjusted to a concentration of 10^6 CFU/ml using a spectrophotometer set at 650 nm.

AmB and oxygen exposures

Amphotericin B was purchased from Sigma Chemical Co. (St. Louis, MO). The formulation of AmB contained sodium deoxycholate (35%) to facilitate the dispersion of AmB in water. AmB was

dissolved in distilled water to yield a 1.0 M stock solution which was filter sterilized. AmB stock solutions were stored under nitrogen at -20°C to prevent auto-oxidation (2,4). Sodium deoxycholate alone (35%) did not affect viability of promastigotes or yeast cells. For Leishmania experiments, AmB stock solutions were diluted to yield final concentrations of 0.1 and 1.0 uM. For yeast cell experiments final concentrations of AmB were 0.25, 0.5, 1.0 and 2.0 uM.

Dissolved oxygen tensions in media with or without AmB were adjusted using vacuum-assisted diffusion (11). Each desired atmosphere (22 mmHg or 150 mmHg) was achieved in a controlled atmosphere glove box (Labconco Corp., Kansas City, MO) and confirmed using a model 1100 medical gas analyzer (Perkin-Elmer Corp., Pomona, CA). Media were pipetted into a 0.5 L bottle and dissolved gases were removed using a vacuum pump. Each desired dissolved oxygen tension was achieved by venting the bottle into the glove box atmosphere. The vacuum/venting procedure was repeated 12 times over a 5 min period. Achievement of the target oxygen tension was confirmed by injecting deoxygenated media into a model 170 pH/blood gas analyzer (Corning Medical, Medfield, MA).

Experimental design

After each target oxygen tension was achieved in the culture media, one part promastigote or yeast cell suspension was added

to nine parts deoxygenated media with or without AmB. The final concentrations of promastigotes and yeasts cells were 1 X 10^6 /ml and 1 X 10^5 CFU/ml, respectively. For Leishmania experiments, five ml aliquots were pipetted into 25 cm^2 tissue culture flasks. Depth of the culture media in the flasks was approximately 3 mm to facilitate gas diffusion. Flasks were incubated in air (21% O_2, 101.3 kilopascals) or hypoxia (5% O_2, 101.3 kPa) at 27°C±1°C for 2 h. Cultures were maintained in controlled atmosphere culture chambers (Bellco Inc., Vineland, NJ). Five independent experiments were run in triplicate for each concentration of AmB. After incubation, promastigote suspensions were removed from flasks, pelleted, and washed with PBS. Promastigotes were then stained with 5 ug/ml fluorescein diacetate for 5 min. Viable organisms stained green and were counted on a hemacytometer using an epifluorescence microscope.

For yeast experiments, 100 ul of yeast cell suspension was added to 900 ul of media containing different AmB concentrations in wells of tissue culture plates (24 wells/ plate). Cultures were incubated in the same hypoxic or normoxic conditions as used for Leishmania experiments, except that yeast cultures were incubated at 35°C. After incubation, ten-fold dilutions of each yeast cultures with or without AmB were made and 100 ul of each dilution was plated in triplicate onto Sabouraud dextrose agar plates. Plates were incubated for 24 h and colony forming units were counted. Five independent experiments were performed.

Confirmation of FDA staining procedure

To verify that the FDA stain was reliable under hypoxic conditions, suspensions of promastigotes which failed to stain with FDA were placed in fresh media and maintained in a normoxic environment (PO_2 = 21 kPa) for 72 hours. In these cultures, no viable organisms were seen, i.e. cellular swelling was present in all organisms and no flagellar motility was observed.

Statistical analysis

Student's t test (paired) was used to compare differences between treatment groups. P values of <0.05 were considered significant.

Results

Effects of hypoxia on AmB-induced killing of Leishmania promastigotes

When Leishmania braziliensis panamensis promastigotes were exposed to AmB under hypoxic or normoxic conditions, no differences between groups were observed. After two h incubation at 27°C, 42% of the original inoculum treated with 0.1 uM AmB in air had survived (Fig. 1). Under hypoxic conditions, only 22% of the original inoculum of promastigotes treated with 0.1 uM AmB survived. These findings were not significantly different. When organisms were exposed to 1.0 um AmB in air or hypoxia for two h, almost all of the promastigotes (99.8 %) were killed (Fig. 1).

Effects of hypoxia on AmB-induced killing of Candida albicans cells

Yeast cells were killed in a dose-dependent manner during a two h exposure to AmB in air. As shown in Fig. 2, 0.25 uM AmB killed 33%, 0.5 uM killed 73%, 1.0 uM killed 97%, and 2.0 uM killed 98.5%. A trend toward decreased AmB-induced killing of C. albicans in hypoxia was observed (Fig. 2). However, statistical significance between air and hypoxia groups was achieved only for the 0.5 uM concentration of AmB.

Discussion

The site of action of AmB is sterol molecules, especially ergosterol, located in cellular membranes of protozoa and fungi. AmB adheres tightly to membranes of susceptible organisms and is not found in the cytoplasm of cells (12). AmB combines with sterol molecules to form aqueous pores in the cell membrane (13). Formation of these pores in the cell membrane results in a loss of ions, especially K^+, and small essential metabolites. Membrane permeabilization is associated with pre-lethal events. AmB concentrations of 0.3 uM or less cause release of small molecules and growth inhibition in L. donovani promastigotes (14). AmB in concentrations greater than 0.5 uM completely inhibit glucose-stimulated respiration and cause cell death in the same organisms (14). Lethal events induced by AmB have been shown to be oxidant-dependent (1,3).

Leishmania promastigotes and yeast cells are biochemically related (13) and appear to be very similar in their response to changes in oxygen tensions and in their susceptibility to AmB. In the current study, hypoxia did not affect the growth of either L. braziliensis panamensis promastigotes or C. albicans cells. In addition, both Leishmania and yeasts also have a high tolerance to the oxygen toxicity, even though their antioxidant defense systems are somewhat different. Leishmania promastigotes lack catalase and glutathione peroxidase, but do have large amounts of superoxide dismutase (15). Both types of organisms are susceptible to the effects of AmB. In this study, almost all of the promastigotes and yeast cells were killed by exposure to a dose of 1.0 uM AmB for two hours. In both our previous work with Leishmania (9) and the study done by Gudewicz et al. with C. albicans (10), a PO_2 of approximately 1700 mmHg was required before additive effects were seen between AmB and HBO.

The results of this study show that tissue level hypoxia does not significantly decrease the efficacy of AmB in killing either Leishmania promastigotes or intact Candida yeast cells. A trend for decreased efficacy of AmB in hypoxic conditions was seen, however, for both organisms. In the case of L. braziliensis panamensis, there was an insignificant mean difference of 20% between air and hypoxic groups at 0.1 uM AmB (42% alive vs 22% alive). In the case of C. albicans yeast cells, there was a significant mean difference of 30% between air and hypoxic groups

at 0.5 uM AmB (56% alive vs 26% alive). However, the mean difference of 33% seen between air and hypoxic groups at 1.0 uM AmB, was not statistically significant.

Although a trend for decreased efficacy of AmB in hypoxic conditions was seen in our experiments, these differences were not significant. Hypoxia had little effect on AmB-induced killing of Leishmania or C. albicans when compared to the results obtained using yeast protoplasts (1) and human erythrocytes (16). AmB-induced lysis of both yeast protoplasts (1) and RBCs (16) were markedly reduced under hypoxic conditions as compared with lysis in air. Relative hypoxia [40 mmHg] reduced lysis of yeast protoplasts to 20% from the 100% seen in air [160 mmHg] (1). Time for 50% lysis of human erythrocytes was significantly increased (64%) under hypoxic conditions (PO_2 = 20 mmHg) at 5.0, 10.0, and 15.0 ug/ml AmB as compared to lysis in normoxic conditions (PO_2 = 150 mmHg). Increases in lipid peroxidation and percent hemolysis of RBCs were seen with concomitant increases in AmB concentration (16). AmB also induces lethal effects in Trypanosoma cruzi which are protozoan parasites closely related to Leishmania. However, evidence of lipid peroxidation of cell membranes was not found in T. cruzi epimastigotes (2). Decreases in free fatty acids and unsaturated fatty acids were consistent with AmB inhibition of fatty acid desaturase activity, not lipid peroxidation. AmB-induced lysis of protoplasts and RBCs may be due to the effects of lipid peroxidation, while lethal effects of AmB seen in

Leishmania and C. albicans could be due to another mechanism.

Acknowledgements

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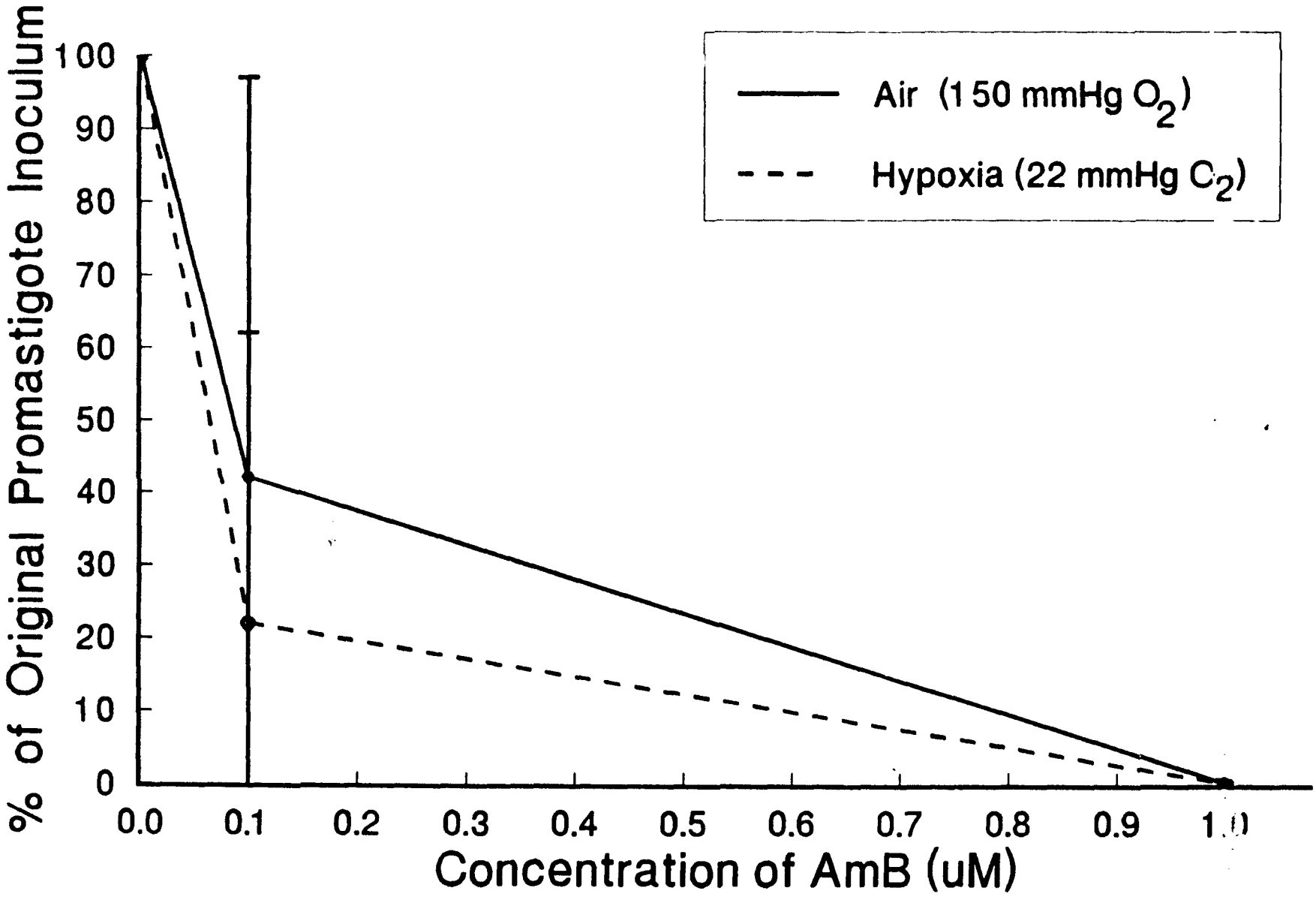
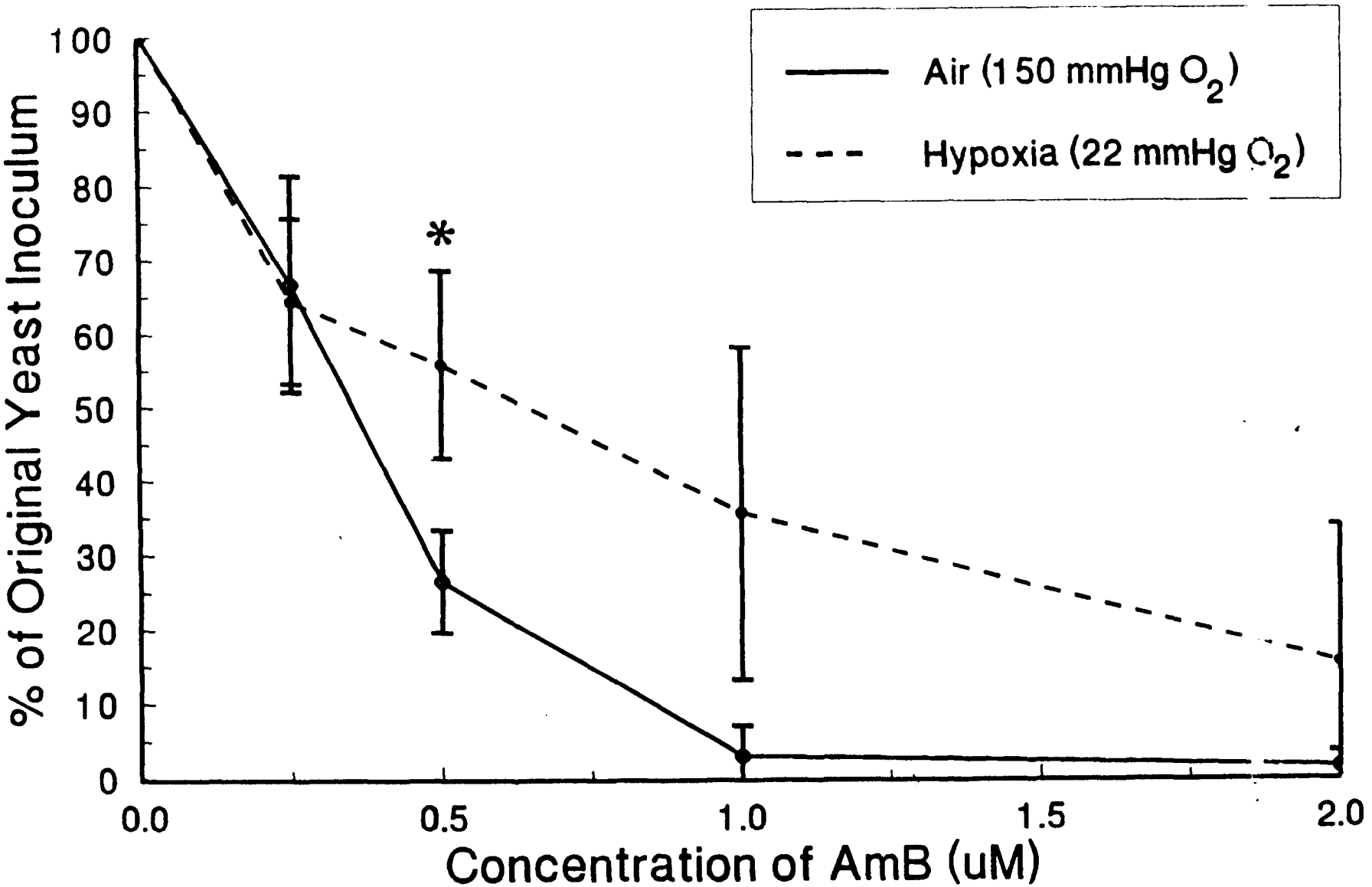
Figure legends

FIG. 1--Survival of L. braziliensis panamensis promastigotes incubated with AmB (0.1 or 1.0 uM) in air or hypoxia for 2 h at 27°C. Each data point represents the mean±SD percent of the original inoculum for five independent experiments done in triplicate.

FIG. 2--Survival of C. albicans cells incubated with AmB (0.25, 0.5, 1.0, or 2.0 uM) in air or hypoxia for 2 h at 35°C. Each data point represents the mean±SD percent of the original inoculum for five independent experiments done in triplicate.


% of Original Promastigote Inoculum
100
90
80
70
60
50
40
30
20
10
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Concentration of AmB (uM)
Air (150 mmHg O_2)
Hypoxia (22 mmHg O_2)


Air (150 mmHg O_2)
Hypoxia (22 mmHg O_2)
*
% of Original Yeast Inoculum
100
90
80
70
60
50
40
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20
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0.0
0.5
1.0
1.5
2.0
Concentration of AmB (uM)